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*THE ENZYMATIC METHYLATION OF RNA AND DNA, VIII. EFFECTS OF BACTERIOPHAGE INFECTION ON THE ACTIVITY OF THE METHYLATING ENZYMES**

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We, as well as others, have previously reported on the presence in *Escherichia coli* of several enzymes which catalyze the transfer of methyl groups from *S*-adenosylmethionine to sRNA,[2-4] ribosomal RNA,[5, 6] and DNA.[4, 7] Although the biological function of the methylated bases which these enzymes produce is still obscure, the species and strain specificity of the methylation reactions suggest that they provide a basis for a recognition mechanism. The virulent bacteriophage-host cell system is an example of a phenomenon involving recognition by the host of a foreign nucleic acid; in some instances, phage DNA is rapidly synthesized while the host DNA is rapidly degraded. If methylated bases are involved in controlling such a recognition mechanism, then a study of the methylated base content of DNA's of various bacteriophages grown in different hosts might provide a clue as to the biological function of the methylating enzymes. In order to establish a suitable system for further investigation, we have studied the effects of phage infection on the activities of the various methylating enzymes in the host cell. This communication summarizes such studies. It has been found that while the RNA methylases are apparently unchanged, DNA methylation activity increases markedly after infection with T2. In contrast, T3 infection induces an enzyme which cleaves *S*-adenosylmethionine to thiomethyladenosine and homoserine.

Materials and Methods.—(a) *Bacteria and phage:* *E. coli* B, used for infection experiments with the T series of bacteriophage was a strain obtained from Dr. C. Bresch of the University of Cologne. *E. coli* K12 strain W3104 and its lysogenic variant, W3104 (λ) were obtained from Dr. A. D. Kaiser of Stanford University and were used for studies on the effects of infection with or induction of bacteriophage λ, respectively.

Phages T1, T2, T4, T5, and T6 were generously provided from the stocks of the Department of Microbiology, New York University School of Medicine. Phages T3 and T7 were gifts of Drs. R. Latarjet of the Pasteur Institute and C. Bresch, respectively. Bacteriophage λ stocks were prepared from a single plaque isolated after plating the supernatant medium of an *E. coli* K12 (λ) culture on *E. coli* K12.

All cultures were prepared in a medium of the following composition (gm per liter): NH_4Cl, 2; $MgSO_4$, 0.3; $CaCl_2$, 0.01; casamino acids "Difco," 10; and glucose, 10. Added separately, after autoclaving, were (ml per liter): 0.5 *M* Na_2HPO_4, 45; and 0.5 *M* KH_2PO_4, 15. Phage lysates were prepared by inoculating exponentially growing cells at a density of 2–5 $\times$ 10^8 per ml with phage at multiplicities of approximately 5 phage per bacterium. The infected cultures were vigorously aerated at 37° until lysis occurred. Phage titers varied from 7 $\times$ 10^9 per ml for phage λ to 5 $\times$ 10^{10} per ml for T-even phages.

(b) *Conditions of infection:* Bacterial cultures (500 ml) were grown in 2-liter Erlenmeyer flasks with vigorous shaking at 37° until a cell density of 5 $\times$ 10^8 per ml was attained; this was determined from absorbancy measurements of the culture at 650 mμ in the Coleman spectrophotometer. Enough phage lysate (warmed to 37°) was then added to give multiplicities of infection of 5–15. The percentage of uninfected cells was determined by diluting a sample of the infected culture 10^4-fold in cold saline 1 min after the lysate was added and plating on nutrient agar at a final dilution of 10^{-6}. For T2, about 5% of the cells escaped infection after 1 min under these conditions and for T3 less than 1% of the cells remained uninfected.

Cultures of *E. coli* K12 (λ) were induced[8] with mitomycin C at a final concentration of 1 μg per ml. This treatment resulted in complete lysis of the culture after about 100 min.

(c) *Preparation of cell-free extracts:* At various times after infection, 150–200 ml aliquots of the culture were withdrawn and quickly cooled by the addition of ice or by swirling in flasks immersed in a dry ice-alcohol bath. Subsequent operations were carried out at 0–5°. After centrifugation at 10,000 $\times$ *g* for 15 min, the bacterial pellets were washed by resuspending in 5 ml of a solution containing 0.5% NaCl and 0.5% KCl. The pellets were again collected by centrifugation and resuspended in 0.5–1.0 ml of a solution containing 0.02 *M* Tris buffer, pH 8.0, 0.01 *M* $MgCl_2$, 0.002 *M* EDTA, and 0.01 *M* 2-mercaptoethanol and transferred to plastic tubes. The bacterial suspensions were disrupted by immersing the tubes in water in the cup of a Raytheon 10-kc sonic oscillator operated at full power for 10–20 min, centrifuged at 40,000 rpm in the Spinco Model L ultracentrifuge for 10 min, and the supernatant fluid retained for enzyme assays. All extracts were stored at 0–5° and not frozen.

(d) *Enzyme assays:* (i) *sRNA methylation:* Reaction mixtures (0.25 ml) contained Tris buffer, pH 8.0, 25 μmoles; $MgCl_2$, 1 μmole; 2-mercaptoethanol, 4 μmoles; C^{14}-CH_3-*S*-adenosylmethionine (3.8 $\times$ 10^7 cpm per μmole), 10 mμmoles; methyl-deficient *E. coli* or *Micrococcus lysodeikticus* sRNA, 308 or 350 mμmoles or nucleotides, respectively, and cell-free extract. After 30 min at 37°, the reaction was terminated and the amount of methyl group incorporated into sRNA assayed as previously described.[9]

(ii) *Ribosomal RNA methylation:* Reaction mixtures (0.25 ml) contained triethanolamine-HCl buffer, pH 8.8, 15 μmoles; 2-mercaptoethanol, 4 μmoles; ammonium sulfate, pH 8.5, 15 μmoles; C^{14}-CH_3-*S*-adenosylmethionine, 10 mμmoles; methyl-deficient *E. coli* ribosomal RNA, 500 mμmoles of nucleotides, and cell-free extract. The addition of cell-free extract was made last. After 30 min at 37°, reaction mixtures were treated as described for the measurement of sRNA methylation.

(iii) *DNA methylation:* Reaction mixtures (0.25 ml) contained Tris buffer, pH 8.0, 25 μmoles; 2-mercaptoethanol, 4 μmoles; C^{14}-CH_3-*S*-adenosylmethionine, 10 mμmoles; methyl-deficient *E. coli* or *M. lysodeikticus* DNA, 100 mμmoles of nucleotides and cell-free extract. After 30 min, the amount of C^{14}-methyl group incorporated into DNA was measured as previously described.[10]

(iv) *RNA polymerase:* Reaction mixtures (0.50 ml) contained potassium maleate buffer, pH 7.5, 25 μmoles; $MnCl_2$, 2 μmoles; 2-mercaptoethanol, 2 μmoles; spermine, 0.5 μmole; ATP, UTP, and GTP, 80 mμmoles each; C^{14}-CTP (1.2 $\times$ 10^7 cpm per μmole), 45 mμmoles; calf thymus DNA, 32 mμmoles of nucleotides, and crude cell-free extract. After 10 min at 37°, reactions were stopped and assayed as previously described.[11]

(v) *Cleavage of S-adenosylmethionine:* Reaction mixtures (0.25 ml) contained Tris buffer, pH 8.0, 25 μmoles; 2-mercaptoethanol, 4 μmoles; C^{14}-CH_3-*S*-adenosylmethionine, 10 mμmoles, and cell-free extract. After 30 min at 37°, the reaction mixtures were chilled; aliquots (0.05 ml) were removed, diluted to 1.0 ml with 0.01 *M* potassium phosphate buffer, pH 7.0, and analyzed by the method of Mudd.[12] The diluted sample was immediately placed on a column (1 $\times$ 3 cm) of XE-64 resin equilibrated with 0.01 *M* phosphate buffer, pH 7.0, and washed with 25 ml of the same buffer. *S*-adenosylmethionine was then eluted with approximately 25 ml of 4 *N* acetic acid.

Other Methods.—Protein was determined by the method of Bücher.[13] Ribose was determined by the orcinol method[14] with a 60-min heating period with adenosine as a standard.

Results.—Measurements of methylation activity after infection of *E. coli* B with various phages are shown in Table 1. There was no significant difference in the

TABLE 1

EFFECTS OF PHAGE INFECTION ON ENZYME ACTIVITIES IN *E. coli*

Cell extract source	Time after infection, min	RNA polymerase	Methylation of sRNA	Methylation of rRNA	Methylation of DNA
Uninfected *E. coli* B	...	12.8	2.2	0.39	0.8
T1-infected *E. coli* B	10	7.9	1.8	0.41	2.4
T2-infected *E. coli* B	12	11.1	1.4	0.29	38.0
T3-infected *E. coli* B	10	6.3	<0.02	<0.02	<0.01
T4-infected *E. coli* B	12	8.4	1.4	0.49	3.6
T5-infected *E. coli* B	15	10.5	1.4	0.29	0.7
T6-infected *E. coli* B	12	7.1	1.6	0.41	0.5
T7-infected *E. coli* B	10	8.2	1.5	0.46	0.7
Uninfected *E. coli* K12	...	...	2.3	2.4	0.3
λ *E. coli* K12	30	...	1.6	1.5	0.4
Uninduced *E. coli* K12 (λ)	...	...	4.2	2.0	0.5
Induction of λ with mitomycin C	60	...	2.4	1.8	0.4

Extracts were prepared and assays performed as described in the text. sRNA and ribosomal RNA were isolated from "methyl-deficient" *E. coli*, except in the case of experiments reported with *E. coli* K12 where ribosomal RNA from *M. lysodeikticus* was used, and DNA was from *M. lysodeikticus*. No significant changes in activity after infection were observed with nucleic acid preparations as methyl group acceptors from either source. RNA polymerase activities are expressed in terms of mμmoles of C^{14}-CMP incorporated in 20 min per mg of protein. Methylation activities are expressed as mμmoles of C^{14}-methyl groups incorporated into an acid-insoluble form in 30 min per mg of protein.

levels of activity of methylation of sRNA and ribosomal RNA with cell-free extracts prepared from any phage-infected cell except that obtained after T3 infection; in this case, both activities were markedly reduced. DNA methylation was also greatly reduced after T3 infection. In contrast, RNA polymerase activity was not markedly altered by infection with T3.

DNA methylation activity increased after infection with phages T1, T2, and T4; the increase after T2 infection, however, was greatest and has varied between 30- and 100-fold, depending on the conditions of infection and the method used to prepare cell-free extracts. The other phages produced no significant changes in DNA methylation activity.

TABLE 2

EFFECT OF TIME ON DNA METHYLATION AFTER PHAGE INFECTION

Extracts of cells infected with	Time after infection, min	DNA methylation, mμmoles C^{14}-CH_3 groups/30 min/mg protein
T2	0	0.3
	2	14.5
	4	27.5
	6	33.7
	15	38.8
T3	0	0.30
	2	0.16
	4	<0.01
	6	<0.01
		μμmoles C^{14}-CH_3 groups/30 min
T2	15	196
T3	6	<1
T2 and T3	...	3

Assays for DNA methylation were as described in the text. In the mixing experiments, the following amounts of extract (in μg of protein) were added: T2-infected 13, T3-infected extract 40; the extracts were prepared from cells infected for 15 and 6 min, respectively.

Cell-free extracts, prepared at various times after infection with T2 and T3, were used to determine the change in DNA methylation activity during the course of the latent period (Table 2). By 6 min after T2 infection, this activity had increased more than 100-fold. Mixing extracts from uninfected cells with extracts from T2-infected cells resulted in additive activities suggesting that T2 infection has not inactivated an inhibitor present in excess in normal cells.

Also shown in Table 2 is the decline in activity after T3 infection. In some ex-

periments virtually no activity could be detected as early as 1.5 min after infection. In this case, however, extracts of T3-infected cells markedly inhibited the activity present in extracts of T2-infected cells. Similar inhibition was observed when extracts of T3-infected cells were mixed with extracts of uninfected *E. coli* B or purified enzyme from *E. coli* W.

TABLE 3

CLEAVAGE OF *S*-ADENOSYLMETHIONINE BY EXTRACTS OF UNINFECTED AND PHAGE-INFECTED *E. coli*

Cell extract source	*S*-Adenosylmethionine cleaved, mμmoles in 30 min/mg of protein
E. coli B	3
T2-infected *E. coli* B	3
T3-infected *E. coli* B	10,000

These values were obtained from the chromatographic data shown in Fig. 1. In all cases, there was a quantitative recovery of radioactivity from the column.

These results indicated the presence of a potent inhibitory factor of DNA methylation in extracts of T3-infected bacteria. Nuclease activity of such magnitude was ruled out by the relative constancy of RNA polymerase activity in these cells. It was found, however, that in reaction mixtures containing extracts of T3-infected cells, *S*-adenosylmethionine, the methyl donor, had virtually disappeared, with the formation of a new compound (Fig. 1). In reaction mixtures containing either no

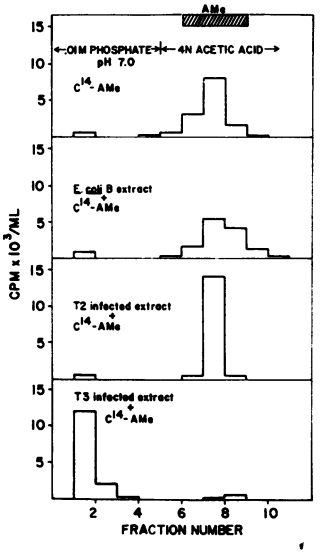

FIG. 1.—Chromatography of reaction mixtures on XE-64 ion exchange resin.[8] Incubations and chromatography were performed as described in the text with the following amount of cell extract (in μg of protein): *E. coli* B 53, T2-infected 66, and T3-infected 13.2. The cross-hatched area at the top of the figure represents the ultraviolet-light-absorbing material of unlabeled authentic *S*-adenosylmethionine (abbreviated AMe) added to the column as a marker.

cell extract or extracts of uninfected or T2-infected bacteria, nearly all of the added *S*-adenosylmethionine was recovered. With extracts of T3-infected cells, however, over 97 per cent of the radioactivity initially present in *S*-adenosylmethionine was eluted from the column by dilute neutral buffer, indicating that the compound had been converted from a strongly positively charged sulfonium salt to a neutral or negatively charged molecule. Table 3 summarizes the rate of cleavage of *S*-adenosylmethionine by cell extracts obtained from uninfected, T2-, and T3-infected bacteria.

Analysis of the product (Table 4) formed from *S*-adenosylmethionine in T3-infected cell extracts revealed that it contained the C^{14}-labeled methyl group, ribose, and a compound with the ultraviolet absorption spectrum of adenine (at pH 7, the absorbancy ratios were 280/260 = 0.19, 250/260 = 0.78) in equimolar quantities. These properties, together with the behavior of this compound on XE-64

ion exchange resin, suggested that the compound was thiomethyladenosine. Homoserine was identified as the other product by paper chromatography in *n*-butanol-acetic acid-H_2O (60:15:25), 77 per cent ethanol, and phenol saturated with 6.7 per cent citrate and 3.7 per cent KH_2PO_4 solution. A ninhydrin reacting substance was present which gave the following R_f values, respectively, in these solvents: 0.34, 0.36, and 0.46. In each case, the R_f value corresponded exactly to that of authentic homoserine.

TABLE 4

C^{14}-*S*-ADENOSYLMETHIONINE CLEAVAGE PRODUCT

	Method of analysis	Amount present, mμmoles per ml
1.	Radioactivity	0.046
2.	Absorbancy	0.042
3.	Orcinol	0.045

The values listed are given for the peak tube of an XE-64 chromatogram of an incubation mixture (2.5 ml) containing Tris buffer, pH 8.0, 250 μmoles; 2-mercaptoethanol, 40 μmoles; C^{14}-CH_3-*S*-adenosylmethionine, 0.9 μmoles (4.3×10^6 cpm per μmole; and 66 μg of protein of an extract of *E. coli* B prepared 10 min after infection with T3. After 30 min at 37°, the mixture was chilled and diluted to 10 ml with 0.01 *M* potassium phosphate buffer, pH 7.0. Chromatography on XE-64 was carried out with 5 ml of this solution on a column with dimensions 1 cm × 7 cm. The analysis of the material in the peak eluted with phosphate buffer was carried out by radioactivity measurement from the known specific activity of the C^{14}-CH_3-*S*-adenosylmethionine; the ultraviolet absorbancy at 259 mμ (assuming a molar absorbancy coefficient of 15.4×10^3 at pH 7.0); and by the orcinol test using adenosine as a standard.

TABLE 5

STOICHIOMETRY OF C^{14}-*S*-ADENOSYLMETHIONINE CLEAVAGE

Compound analyzed	Amount utilized or formed, μmoles
C^{14}-*S*-adenosylmethionine	−0.63
C^{14}-thiomethyladenosine	+0.57
Homoserine	+0.60

Reaction mixtures (0.4 ml) containing Tris-HCl buffer, pH 8.0, 50 μmoles, 2-mercaptoethanol, 8 μmoles, C^{14}-CH_3-*S*-adenosylmethionine, 1 μmole, (3.8×10^5 cpm per μmole) and 100 μg of protein of a T3-infected extract of *E. coli* B prepared as described above were incubated at 38° for 1 hr. The reaction was stopped by the addition of 1 ml of absolute ethanol, and the protein was removed by centrifugation. After adjusting the pH of the solution to 5.0, the resulting concentrated solution was spotted on Whatman 3MM filter paper and chromatographed in *n*-butanol-acetic acid-water (60:15:25). *S*-adenosylmethionine and thiomethyladenosine were located by ultraviolet light, and homoserine after reaction with ninhydrin. All the compounds were eluted from paper with water. Homoserine was estimated by the quantitative ninhydrin method according to the procedure of Rosen,[15] and *S*-adenosylmethionine and thiomethyladenosine were quantitated by radioactivity measurements.

The conversion of *S*-adenosylmethionine to thiomethyladenosine and homoserine by extracts of T3-infected cells was stoichiometric (Table 5).

Discussion.—Following infection of *E. coli* B with phage T2, there is an increase in DNA methylation activity which begins almost immediately. Pending further purification of the enzyme or enzymes involved, it cannot be determined whether the increase in activity is due to *de novo* synthesis of a phage-directed protein or whether phage infection alters the activity of the pre-existing host enzyme. It is noteworthy that while phage T2 brings about a dramatic increase in DNA methylation activity and T4 shows a small increase, T6 infection produces no change at all. The absence of significant quantitative changes in RNA methylating activities is, however, no indication that the enzymes responsible remain unaltered after infection.[16]

The striking effect of T3 infection on methylation of DNA and RNA can be related to the appearance of a potent *S*-adenosylmethionine hydrolyzing enzyme. This enzyme appears to be similar to the enzymes present in yeast[12] and bacteria.[17] It has been reported that *E. coli* B does not normally contain this activity,[17] and our results agree with these observations. We can detect little or no activity in extracts of uninfected cells. It is also of interest that infection with T7, which is closely related to T3, does not produce similar effects. The increase in activity

of S-adenosylmethionine hydrolase after T3 infection may prove a useful tool in the study of the physiological aspects of infection with these two phages.[18]

Glover *et al.*[19] have recently reported that under certain conditions T3 undergoes restriction but not modification. In view of the possible relationship between host-induced modification and methylation of DNA, the finding that T3 infection brings about a marked increase in an enzyme which destroys S-adenosylmethionine (the methyl donor) may indicate a role for this enzyme in the modification phenomenon.

Summary.—Infection of *Escherichia coli* B with bacteriophage T2 leads to a more than 100-fold increase in the rate of methylation of DNA by cell-free extracts. Smaller increases are seen after infection with phages T1 and T4. No changes in the methylation of soluble RNA and ribosomal RNA are observed. Infection with T3 results in the appearance of an enzyme which cleaves S-adenosylmethionine, the methyl group donor in nucleic acid methylation. The products of this cleavage appear to be thiomethyladenosine and homoserine which are found in equivalent amounts.

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